

## LOSS OF LIMBS AS A STIMULUS TO ECDYSIS IN BRACHYURA (TRUE CRABS)<sup>1</sup>

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The common means of inducing precocious molts in Crustacea is by eyestalk extirpation (Zeleny, 1905), which removes the X-organ-sinus gland complex containing the postulated molt inhibitory hormone (MIH; Passano, 1960) and presumably allows the action of ecdysterone to initiate molt preparations. This procedure, though effective in stimulating molting in many species, frequently kills the animals either at the time of surgery or at the time of ecdysis itself. Such is the case with one of the experimental animals used in our laboratory, the land crab, *Gecarcinus lateralis*. Moreover, the injection of ecdysterone into unoperated animals frequently leads to the death of the animals even in species where it is an effective stimulus to molting (Krishnakumaran and Schneiderman, 1968; Williams, 1968; Skinner and Graham, 1970). Previously we reported that in *Gecarcinus* the loss of a large number of limbs, either pereopods (walking legs) or chelipeds (claws), triggered precocious but apparently physiologically normal molts with high survival, whereas injections of ecdysterone were ineffective (Skinner and Graham, 1970).

It is known that regeneration of lost limbs occurs in the premolt period of the land crab (Bliss, 1956; Hodge, 1956a, 1956b; Skinner, 1958, 1962; Skinner and Graham, 1970) and many other Crustacea (Emmel, 1910; Bliss, 1960; Needham, 1965; Hay, 1966; Goss, 1969). Furthermore, the loss of numerous limbs triggers precocious molts in certain insects (Cameron, 1927) and the land crab (Bliss, 1956; Skinner and Graham, 1970). On the other hand, in some cases such as the cockroach, *Blattella*, loss of limbs before a certain critical time can as much as double the interval before the next ecdysis (O'Farrell, Stock and Morgan, 1956).

This paper represents a study of the interrelationships between molting and limb regeneration in the Brachyura, or true crabs. It includes observations on: (1) the effect of limb loss on the duration of the molt cycle of several species of marine Crustacea, with data on precocious molts caused by eyestalk removal included for comparison; (2) the minimal number of legs which must be removed to cause molting; (3) the effect of the total number of legs removed on the size of the subsequent limbs regenerated (regenerates); (4) the effect of lack of privacy and of light regimen on the duration of the molt cycle (Bliss and Boyer, 1964);

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(5) the interaction between loss of regenerating limb buds in the early premolt period, duration of the premolt period, and re-regeneration of the lost regenerates. Experiments in (2), (3), (4) and (5) used the land crab, *Gecarcinus lateralis*, as the experimental animal.

#### MATERIALS AND METHODS

Specimens of the land crab, *Gecarcinus lateralis*, were maintained in the usual manner (Skinner, 1962). Specimens of two portunoid crabs, (the blue crab, *Callinectes sapidus*, and the green crab, *Carcinus maenas*) the fiddler crabs, *Uca pugnax* and *U. pugilator*, and the spider crab, *Libinia emarginata*, were kept in sea tables with running water at the Marine Biological Laboratories, Woods Hole, Massachusetts. They were fed mussels.

Animals were caused to autotomize limbs (Wood and Wood, 1932) by cutting at the merus. Eyestalks were removed by cutting at the articulating membrane. To increase survival, one eye was removed on day 1, the other on day 2 or later.

A single leg was removed from control or eyestalkless specimens to permit us to detect an approaching molt by the progress of limb regeneration (Bliss, 1956; Skinner, 1962, 1965). Experiments on *Gecarcinus* were performed throughout the year; experiments on the marine crabs were performed in mid-June through August.

The size of limbs was determined by measuring the external dimensions of the various segments as well as by weighing the limbs. Postmolt limb weights were determined one month after ecdysis, since the limb immediately following ecdysis contains only some 17% of the final intern molt quantity of tissue isolable one month later (Skinner, 1966a) after a period of rapid protein synthesis has occurred (Skinner, 1966b).

#### RESULTS

##### *Response of marine Crustacea to limb loss*

*Libinia*: Neither controls (20), eyestalkless (8) nor animals with 6 or 8 legs missing (L. A. = legs autotomized animals; 20, total) molted or showed any sign of an approaching molt. Scabs remained on the autotomy plane throughout the experiment, which was terminated after 10 weeks (Table I).

*Carcinus*: Four animals missing 6 limbs regenerated legs and molted successfully; two were killed by other animals. No other animals (*i.e.*, eyestalkless or controls) showed any indications of an approaching molt. Eyestalkless specimens did show copulatory behavior typical of premolt animals, with the males grasping the females beneath them.

*Callinectes*: Both eyestalkless animals and those missing 6 limbs prepared for ecdysis with attendant limb regeneration. Of 10 eyestalkless specimens, all survived for several weeks after eyestalk removal, 3 survived until the late premolt period but only one until ecdysis (42 days after eyestalk loss). That animal died after having split the epimeral suture in the branchiostegite region and initiating emergence from the old exoskeleton. No appendages were freed at the time of death. The mucilaginous layer (molting fluid) was apparent. In the land crab,

TABLE I

*Effect of autotomy of legs or eyestalk removal on the interval to ecdysis of marine crabs*

Animal	Treatment	Effect	Days to ecdysis
<i>Libinia</i> (Spider crab)	E/S* (8)†	0	—
	6 to 8 L. A.‡	0	—
<i>Carcinus</i> (Green crab)	E/S (10)	0	—
	6 or 8 L. A. (6)	+	48 ± 5 (S.D.)
<i>Uca pugnax</i> (Fiddler crab)	E/S (24)	+	22 ± 3
	6 or 8 L. A. (24)	+	25 ± 2
<i>Callinectes</i> (Edible blue crab)	E/S (10)	+	42
	2 C. A.	+	51, 51, 61, 64, 70
	4 L. A. (5)		

\* E/S = Eyestalks removed.

† ( ) = Number of animals in sample.

‡ L. A. = Legs autotomized.

*Gecarcinus*, eyestalkless animals commonly die slightly later, when they have freed themselves almost completely from the old exoskeleton.

The interval to ecdysis was longer in animals triggered to undergo precocious molts by limb loss than in those stimulated by eyestalk extirpation which removes the MIH (Table I). However, the viability of the L. A. animals was considerably greater. In addition to 5 animals that molted, two other groups of 6 and 10 L. A. animals had responded, as witnessed by extensive limb regeneration. Unfortunately, that experiment had to be terminated before the animals underwent ecdyses.

*Uca*: Both *U. pugnax* and *U. pugilator* responded to both treatments. As in the case of *Callinectes*, there was a more rapid response to eyestalk removal, with ecdysis occurring within 20 to 25 days. The loss of 6 or 8 walking legs also stimulated premolt preparations, including limb regeneration. Ecdysis occurred approximately 25 days after limb loss. The mortality of both groups of experimental animals was high, with only one eyestalkless animal surviving ecdysis. Others also report high mortality of eyestalkless *Uca* (Fingerman and Yamamoto, 1967). However, the positive response of the animals to limb loss was clear (See Fig. 1, which shows photographs of *Uca* and other crabs stimulated to molt by limb removal.) As with *Gecarcinus*, removal of one appendage of the marine crustaceans could be useful as an early indication of an approaching molt (Bliss, 1956; Skinner, 1962).

#### *Effect of eyestalk removal as compared to limb loss on precocious molts*

We and others (Passano, 1960; Skinner, 1968) have for years used eyestalk extirpation as a means of propelling various species of Crustacea into precocious molts. The lethargic postoperative behavior of the animals, as well as their demise at the time of the subsequent ecdysis, indicated that although their macromolecular metabolism appeared indistinguishable from that of normal premolt animals (Skinner, 1965, 1966a, 1966b), nonetheless their premolt period was ab-

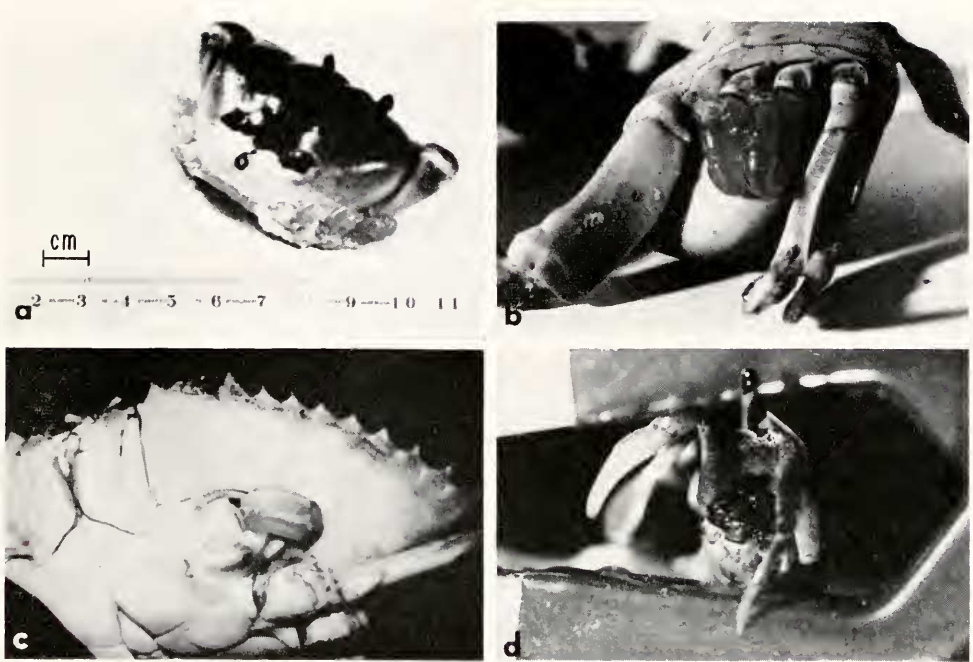


FIGURE 1. Specimens of crabs with regenerating limb buds. (a, b) *Gecarcinus lateralis*, (c) *Callinectes sapidus* and (d) *Uca pugnax*. Eight, 4, 6, and 6 walking legs were removed from animals (a, b) 45, (c) 36, and (d) 20 days previous to the time of photography.

normal. We have performed a series of experiments on *Gecarcinus* to compare the relative effectiveness of eyestalk extirpation and limb loss in inducing precocious molts.

We find that eyestalk removal leads to the most rapid molting preparations (Table I) but is accompanied with 100% mortality at the time of ecdyses (Fig. 2). Molts induced by limb loss, though somewhat delayed in time, almost always lead to healthy, normal animals (Fig. 2). Even those specimens deprived of 8 walking legs, though clumsy in their movements, molt successfully almost 100% of the time. Indeed, in most experiments, we observe no deaths following such treatment.

#### *Minimum number of limb autotomies required to stimulate precocious molting preparations*

These and the remaining experiments in this paper were performed with *Gecarcinus*.

Loss of from 1 to 4 limbs had no effect on the duration of the molt cycle (Table II). Animals which have lost 5 limbs, on the other hand, respond except when kept in community tanks. Loss of from 6 to 8 limbs, including various combinations of chelipeds and pereopods, is almost 100% effective. Since in *Gecarcinus* the chelipeds comprise as much as 35% (21 to 35%; Table III) of the total body weight, whereas the walking legs contribute 11 to 17% (Table III),

TABLE II

*Effect of autotomy of legs on interval to ecdysis in Gecarcinus. Abbreviations are:*  
*L. A. = walking leg autotomized; C. A. = cheliped autotomized; (a) = In*  
*jar, ca. 10 min light/week; (b) = In jar, ca. 10 hr light/week;*  
*(c) = Community tank (6-8 animals); 10 hr light/week*

Treatment	Animals (No.)	Mean time to ecdysis (Days $\pm$ S.D.)
1 L. A. (a)	14	>200
(c) stock supply	>300	>200
2 C. A. (a)	6	>200
4 L. A. (a)	6	>165
5 L. A. (a)	7	86 $\pm$ 17
(b)	8	86 $\pm$ 8
(c)	8	>131
2 C., 4 L. A. (a)	8	77 $\pm$ 25
6 L. A. (a)	8	89 $\pm$ 18
8 L. A. (a)	8	70 $\pm$ 12
(b)	7	81 $\pm$ 17

loss of two chelipeds should have been effective in stimulating molting if the stimulus were loss of tissues mass. However, such was not the case.

The duration of the premolt period is dependent on the size of animals, being

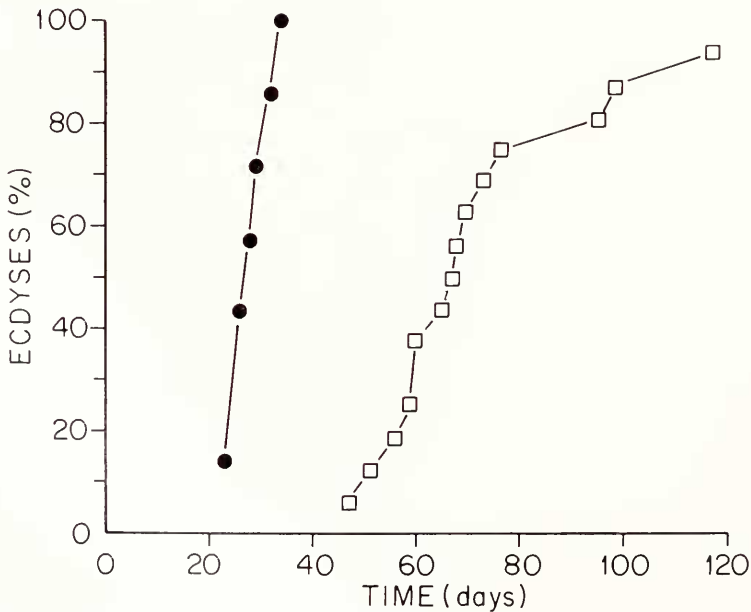


FIGURE 2. Effect of eyestalk removal or autotomy of limbs on the duration of the intermolt period of *Gecarcinus*. Eyestalks were removed on successive days from 7 animals ●—●, all of which died at ecdysis; 8 walking legs were autotomized from 15 animals □—□. The animals were housed in individual bottles and observed until ecdysis occurred.



TABLE III

*Per cent body weight comprised by claws or walking legs of Gecarcinus lateralis.  
Animal 3 was female; all others were males*

	Total weight (Grams)	% Body weight	
		2 Chelipeds	8 Pereiopods
Animal 1	53.0	22	17
2	60.4	22	15
3	63	21	11
4	64.5	27	14
5	67	31	16
6	96	35	13
7	99	32	17

longer for large animals than for small. For example, animals of carapace width 4.2 to 4.7 cm underwent ecdyses 33 to 54 days after limb autotomy, whereas animals of carapace width 5.2 to 5.3 cm took 50 to 82 days. Therefore, in any one experiment, animals of similar size were used.

#### *Environmental effects on the length of the molting cycle*

When crabs lacking either 6 or 8 walking legs are maintained in bottles, which are stored in relatively light-tight cabinets, they receive approximately 10 min of light per week. Such animals molt sooner than those maintained in community tanks that receive 10 to 12 hours of light per week during the routine cleaning and feeding operations (Table II). This observation confirms a previous report of Bliss and Boyer (1964). The following experiment demonstrated that the effect was due to privacy rather than the amount of exposure to darkness. A series of animals was maintained in bottles kept outside the cabinets usually used for storage of experimental animals. Thus these animals received the same exposure to light as did the animals kept in groups of 5 or 6 in community tanks. These animals molted as soon as did animals kept in bottles in the dark (Table II).

It should be noted that the marine crabs studied were maintained in community tanks. Since limb removal stimulated precocious molts in all species of marine animals (*Callinectes*, *Cancer*, *Uca*) except *Libinia*, it may be that *Libinia* is more sensitive to the presence of other animals than the other marine crabs.

#### *Effect of total number of limbs removed on the size of the regenerate*

Regenerated limbs are somewhat smaller than their non-regenerated counterparts. Quantitation of the size difference leads to the following observations: (Table IV) Compared to those of bilaterally symmetrical partners, the weights of non-regenerated limbs taken from 29 animals varied by no more than 4%, whereas the weights of regenerated limbs were smaller by approximately  $\frac{1}{3}$  when from 1 to 6 limbs were removed. Since there are no normal, bilaterally symmetrical limbs left on crabs that have been induced to regenerate 8 walking legs, the weight

TABLE IV

*Effect of the removal of one to eight limbs on the size of regenerated limb. Regenerative load refers to the number of limbs regenerated during a molting period. One or more months after ecdysis, limbs were removed and weighed. Regenerated legs were compared to bilaterally symmetrical non-regenerated legs. To test individual variability, crabs were induced to autotomize a pair of normal limbs. The data on 8 L. A. animals were obtained by comparing the weights of legs of normal animals of the same size (see text)*

Regenerative load (walking legs)	Percentage change in weight between any two walking legs	Sample size
0; (individual variability)	$\pm 4.4$	29
1	-34.5	6
6	-34.7	16
8	-48.4	9

of such legs was compared to the weight of non-regenerated (normal) legs of a series of crabs of the same carapace width, a fixed parameter useful for comparing animals of different stages of the molt cycle. Loss of 8 walking legs leads to a striking decrease in the mass of tissue contained in each limb (Table IV). Rough calculations of the mass of tissue regenerated indicated that an animal (of the sizes used in these experiments) was capable of synthesizing approximately 4 grams of tissue which was distributed among the regenerated appendages. Thus, if 6 limbs were regenerated, each weighed  $\frac{1}{3}$  less than non-regenerated partners whereas if 8 were regenerated, each weighed  $\frac{1}{2}$  that of its non-regenerated control.

*Effect of removal of partially regenerated limbs on the time of ecdysis*

In another series of experiments (Skinner and Beattie, 1971), we have been studying the metabolic and biosynthetic properties of regenerating limbs. For those experiments, we cause the animals to autotomize regenerating limbs at various stages in their development. In one of our early experiments, limb buds of the same external dimensions and appearance were removed from 3 crabs. Two of the crabs molted within 10 days (see Animals 6 and 7, Fig. 3), without regenerating the limbs removed. The third crab, which molted 35 days after the removal of its limb buds, had regenerated those removed (Animal 19, Fig. 3). Apparently, there was a delay in the time of ecdysis sufficient to permit reformation of the limbs.

Since the length of time from one ecdysis to the next is not clearly defined in the land crab as it is for ecdyses specific to particular insect larval instars, it is not possible to determine the magnitude of the retardation in time of ecdysis with certainty. However, in observations on 30 animals (Fig. 3), no animal which underwent ecdysis within 25 days after loss of partially regenerated limbs had replaced those removed. By contrast, all animals which molted 33 or more days after autotomy of limb buds had re-regenerated. Thus the shortest time required for re-regeneration was 33 days after autotomy of partially regenerated limb buds. This corresponds favorably with the time required from eyestalk removal to ecdysis,

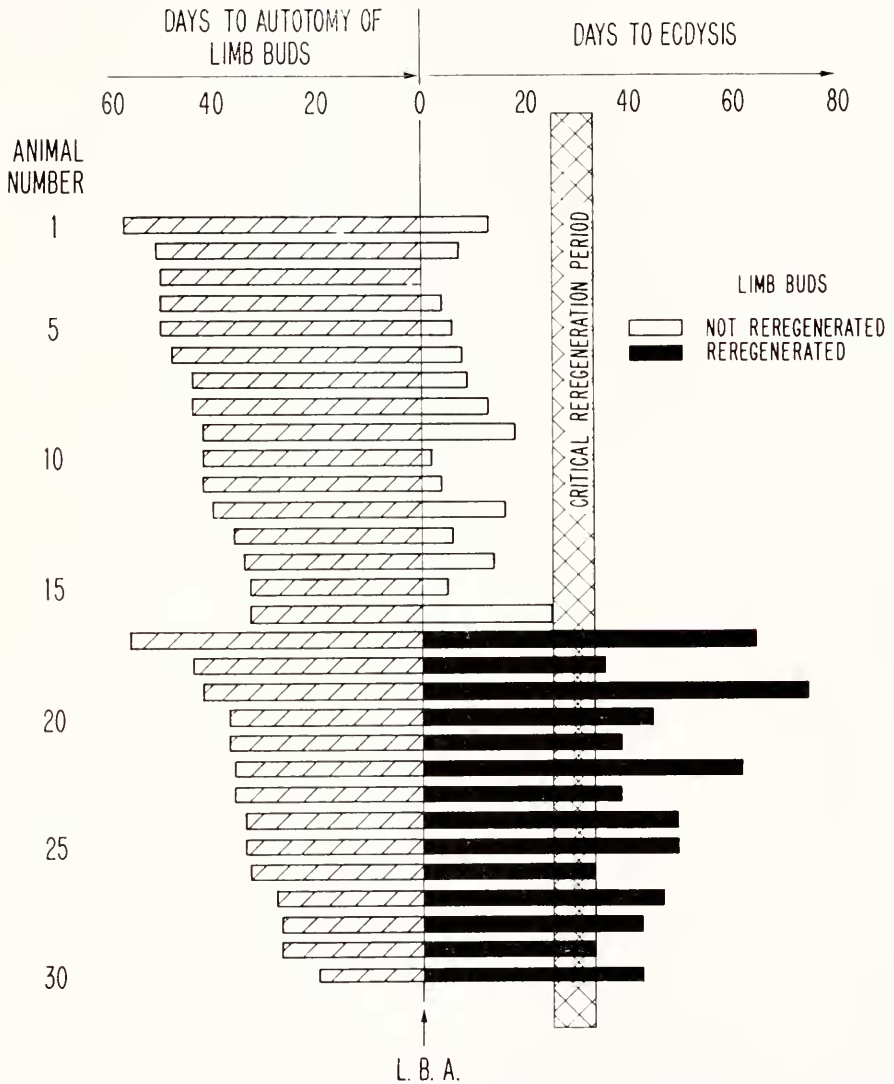


FIGURE 3. Effect of removal of regenerating limb buds on the duration of the premolt period of *Gecarcinus*. Molting preparations were initiated in 30 animals by limb removal and at some later date (Day 0 in this figure) regenerating limb buds were autotomized (L. B. A.). Animals were observed to the next ecdysis. The total length of the horizontal bar represents the duration of the complete premolt period. At the time of the next ecdysis it was noted whether the limbs had been re-regenerated. For purposes of graphical presentation here the animals are ordered into 2 groups: (1) numbers 1 through 16 are those that did not re-regenerate; (2) 17 through 30 are those that did. Within each group, the animals are listed from the longest to the shortest interval between initiation of molt preparations and limb bud autotomy. Note that the duration of the premolt period in animals that did not re-regenerate (Group 1) varied from 38 to 70 days, while in those that did re-regenerate (Group 2) the premolt period varied from 60 to 120 days. The cross hatched bar indicates the critical period (see text).



with attendant limb regeneration (Fig. 2), which was 27 days in animals of similar size.

#### DISCUSSION

We have now demonstrated that the loss of a large number of limbs causes precocious molts not only in the land crab but also in three species of marine crabs. Two of these also prepare for ecdysis soon after eyestalk removal (*Calinectes* and *Uca*) but frequently die at the time of or shortly after ecdysis. The third (*Carcinus*) does not prepare for molting after eyestalk removal if greater than a certain size (approximately 2 cm carapace width, Bauchau, 1961; these data). Our experiments indicate that in large specimens of the green crab, limb loss is a more effective stimulus to ecdysis than is eyestalk removal.

Our experiments on the marine crabs, though preliminary, show that precocious molts triggered by loss of a critical number of limbs lead to apparently normal, healthy ecdyses, which the animals survive with as high viability as from normal ecdyses. Thus, this appears to be the method of choice for inducing precocious molts.

We do not suggest that these experiments demonstrate the presence of a molt-inhibitory factor in the limbs of Crustacea. A more likely hypothesis is that the stimulus to precocious molts is due to the severing of a critical number of nerves.

It would be of interest to attempt to stimulate precocious molts by severing the nerves to the walking legs of crabs. We expect that these experiments would prove to be technically difficult because of the rapidity with which the animals, at least *Gecarcinus*, autotomize limbs when confronted with noxious stimuli, such as anesthetization by exposure to the cold (4° C) or the injection of materials into the base of the appendage.

Cameron (1927) found that the interval between molts in another arthropod, the centipede *Scutigera forca*, was shortened by one half the normal time when *all* its legs were removed. Loss of as many as 26 of the 30 legs was ineffective in shortening the duration of the molting cycle. Unlike *Scutigera*, loss of *all* limbs apparently inhibits molting preparations in *Gecarcinus*. Two of our specimens of *Gecarcinus* lost all of their limbs in combat with other crabs. Both had normal eye reflexes and bore no evidence of other bodily injury. Yet, there were no signs of regeneration after 3 months, although other animals from the same shipment missing 6 or 8 walking legs had long since regenerated their lost limbs and undergone ecdyses. Since specimens of *Gecarcinus* respond within 3 weeks by basal growth of autotomized appendages, and since the limbless animals were hand-fed, it is unlikely that starvation was the explanation for their lack of response, especially since Cameron (1927) found that starved specimens of *Scutigera* survived one ecdysis and lived well into the next intermolt period (approximately 60 days).

A phenomenon similar to the critical period for re-generation of limbs in *Gecarcinus* (Fig. 3) was reported in the insect, *Blatella* (O'Farrell and Stock, 1953). Under conditions when the duration of the first instar was 12 days (25° C), loss of one limb between days 1 and 4 increased the period until the next ecdysis by 3 days. The total duration of the instar was then 15 instead of 12 days. At higher temperatures (30° C), the intermolt duration was increased from 5 to

6 days by the loss of one limb. A limb lost after a certain critical period was not regenerated by the time of the next ecdysis. Rather, it remained as a papilla, to be regenerated during the next premolt period (O'Farrell and Stock, 1953).

The crustacean premolt period may be subdivided into 5 parts, designated stages  $D_0$  through  $D_4$  (Drach, 1939; Skinner, 1962, 1966a). A substantial fraction, if not all, of limb regeneration occurs in  $D_0$  (Skinner, 1962). It has long been known that there is a critical time in the arthropod premolt period (O'Farrell *et al.*, 1956; probably  $D_0$  in crustaceans; Skinner, 1962, 1966a) before which limbs must be removed if they are to be regenerated by the first ecdysis following limb loss. The ability of an animal to prolong  $D_0$ , in which it synthesizes another set of limbs and concomitantly delays the next series of premolt events (such as apolysis, the separation of the epidermis from the old exoskeleton, Jenkin, 1970; resorption of the old exoskeleton and synthesis of a new exoskeleton, Skinner, 1962) points up the complexity of the controls over various premolt phenomena. Our observations indicate that even if a single "on" switch is thrown to initiate the premolt period, the ordered series of events leading to a normal ecdysis do not proceed on an invariant time schedule. The whole premolt process appears to be self-monitored in such a way that subsequent events cannot proceed until early events reach a critical stage of completion and the *duration* of the critical period can be greatly extended. In fact, we think that the animals that did not re-regenerate were already in stage  $D_1$  (and that the epidermis had already separated from the old exoskeleton when their regenerating limb buds were autotomized). Clearly, there is no turning back. The interacting controls are such that the animals did not even initiate the re-regeneration process.

We wish to thank W. G. Beattie and E. Aug for their help with some of these experiments. Some experiments were performed by the senior author in conjunction with the Experimental Invertebrate Course at the Marine Biological Laboratory, Woods Hole, Massachusetts. The help of the collecting staff and students in the course was greatly appreciated, as were the photographs of *Callinectes* and *Uca*, taken by Mr. P. J. Oldham of the Systematics-Ecology Program. D. E. Graham was an Oak Ridge Graduate Fellow under appointment from the Oak Ridge Associated Universities.

#### SUMMARY

1. Loss of 6 to 8 pereopods or chelipeds triggers precocious molts in a number of marine crabs including the green crab, *Carcinus maenas*, the blue crab, *Callinectes sapidus*, and the fiddler crabs, *Uca pugnax* and *U. pugilator*, but not in the spider crab, *Libinia emarginata*. Mortality rates are negligible compared to those of animals induced to molt by eyestalk removal.

2. Precocious molts can be elicited in the land crab, *Gecarcinus lateralis*, by the loss of 5 to 8 appendages (pereopods and chelipeds) but the loss of all 10 appendages inhibits molting. Loss of a cheliped which in *Gecarcinus* may have a mass ten times greater than a pereopod is no more effective than loss of a walking leg.

3. The size of the regenerates formed in *Gecarcinus* is reduced by one-third from normal size when from 1 to 6 pereopods are lost, and by one-half when 8 limbs are regenerated.

4. When one or more partially regenerated limbs is removed before a certain critical time in the premolt period (Stage D<sub>0</sub>?) the animal re-regenerates replacement appendage(s). This results in significant lengthening of the interval before ecdysis occurs.

*Note added in proof:* Dr. George D. Bittner (personal communication) finds that in the crayfish, *Procambarus clarkii*, cutting of the nerves to 6 pereopods is effective in shortening the interval between two ecdyses. In this species nerves to the pereopods can be cut without the induction of autotomy.

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